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Promotional Effects of CO_2 Laser and Scalpel Incision on 4-NQO-Induced Premalignant Lesions of Mouse Tongue

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Background and Objectives: **CO_2 laser and scalpel incision have been demonstrated to have promotional effects on oral neoplastic lesions. However, a precise understanding has not been achieved as to which modality has a more significant effect on cancer promotion. The purpose of this study was to determine the histological and biological changes after CO_2 laser surgery and scalpel incision in oral premalignant lesions.**

Study Design/Materials and Methods: **Premalignant lesions of mouse tongue induced by 4-nitroquinoline-1-oxide (4NQO) in drinking water for 4 months were used in this study. A 2-mm incision was made on the right margin of each mouse tongue, using either a CO_2 laser (group A) or a scalpel (group B). Mice without incisional treatment were used as controls (group C). Seven months after laser and scalpel treatments, hematoxylin-eosin staining and proliferating cell nuclear antigen (PCNA), epidermal growth factor receptor (EGFR), and p53 immunostaining were performed for tongue specimens.**

Results: **The epithelia of right tongue margins showed more severe dysplasia than those of left tongue margins in both group A and group B. The PCNA labeling indices (LIs) and EGFR expression for right tongue margins were also higher than for left margins in both group A and group B. There was no obvious difference between these two groups. Almost no p53-positive staining was found in either group.**

Conclusion: **CO_2 laser surgery and scalpel incision seem to have similar promotional effects on oral premalignant lesions. Lasers Surg. Med. 25:207–212, 1999.** **© 1999 Wiley-Liss, Inc.**

Key words: CO_2 laser; scalpel; promotional effects; premalignant lesions; PCNA; EGFR; p53

INTRODUCTION

Oral premalignant lesions such as leukoplakia and erythroplakia carry the potential risk of malignant transformation. Transformation rates have been estimated to be between 0.13–17.6% [1–5]. The treatment of these lesions has not been standardized, although surgical excision using a laser or scalpel is considered the most effective method [3,6,7]. Nevertheless, the promotion of oral cancer caused by laser and scalpel incision has been reported in previous studies [8–12].

Improved healing and a low complication rate associated with laser surgery have led to increased use of this modality for treatment of oral premalignant lesions [6,7].However, previous studies indicate that laser surgery produced greater release of growth factors and caused sig-

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nificantly greater cancer promotion than did scalpel incision [10,11]. On the other hand, some reports show that there are no significant differences in expression of growth factors at the majority of time points between CO_2 laser wounds and scalpel wounds [13], and the cure rates of 90–97% by using CO_2 laser surgery appear to be higher than those with other techniques [3,7]. Thus, it is not precisely known which modality has a more significant effect on cancer promotion.

Proliferating cell nuclear antigen (PCNA), epidermal growth factor receptor (EGFR), and p53 have been demonstrated as useful biomarkers in oral carcinogenesis [14–21]. In order to determine the change in oral premalignant lesions after laser and scalpel surgery, immunohistological investigation of PCNA, EGFR, and p53 was performed in a mouse model of oral premalignant and malignant lesions induced by 4-nitorquinoline-1-oxide (4NQO).

MATERIALS AND METHODS

Animal Model

A total of 38, 6-week-old, 21–24 g male CBA mice (Charles River, Osaka, Japan) was used in this study. The carcinogen 4NQO (Wako Pure Chemical Industries, Osaka, Japan) was dissolved in tap water to a final concentration of 0.001% and held in a light-shielded bottle. The 4NQO solution was administered orally as drinking water for 4 months.

Laser and Scalpel Treatments

The mice were anesthetized, and a 2-mm incision was made on each right margin of mouse tongue using either a CO_2 laser (NiiC Lasery Model 30Z, Tokyo, Japan, output power 6 W) for a group A ($n = 22$), or a scalpel for group B ($n = 10$), under a surgical microscope. Mice treated only with 4NQO were used as controls (group C, $n = 6$).

Seven months after laser and scalpel treatments, all mice were sacrificed by overdose of pentobarbital sodium. The tongues were excised, fixed with 10% neutral-buffered formalin, and embedded in paraffin. Sections of 4-μm thickness were prepared for hematoxylin-eosin and immunohistochemical staining.

Immunohistochemistry

Anti-PCNA monoclonal antibody (PC10, 1:100 diluted, DAKO M0879, Glastrup, Denmark), anti-EGFR monoclonal antibody (Clone F4, 1:200 diluted, Sigma, St. Louis, MO), and anti-p53 polyclonal antibody (CM1, 1:500 diluted, Medac GmbH, Hamburg, Germany) were used as primary antibodies.

Sections were deparaffinized with xylene and rehydrated through a graded series of ethanol. Endogenous peroxidase was inhibited by treatment with 0.3% H_2O_2 in methanol for 30 min. After washing in phosphate-buffered saline (PBS), the sections were incubated with diluted normal blocking serum for 30 minutes. For EGFR and p53 immunostaining, sections were treated with avidin D and biotin blocking solution (Vector Laboratories, Burlingame, CA) for 15 minutes to block nonspecific binding of biotin/avidin system components. Primary antibody of PCNA was applied for 30 minutes at room temperature, and EGFR and p53 antibodies were applied at 4°C overnight, followed by diluted biotinylated secondary antibody for 30 minutes and avidin-biotin peroxidase complex (ABC) reagent for 30 minutes (Vectastain Elite ABC Kit, Vector Laboratories). Negative control sections were incubated with PBS instead of primary antibodies.

Immunohistochemical reactions were developed with diaminobenzidine (DAB) solution (DAB 20 mg in 100 ml 0.05 M Tris buffer containing 17 μl of 30% H_2O_2). After washing in running water, the sections were lightly counterstained with Mayer's hematoxylin.

Evaluation

Epithelial dysplasia. The histological change of both tongue margins were evaluated, based on the World Health Organization criteria reported elsewhere [5].

PCNA labeling index (LI). The basal and parabasal cells of both tongue margins were counted in every section. All nuclei showing brown staining were considered positive. The PCNA LIs were calculated, and LI and standard deviation (SD) values were determined for each group, and Student's t-test was used to analyze the data.

Grading of EGFR. EGFR was assessed on the basis of a previous study by Grandis et al. [22]. Briefly, all cells of both tongue margins were counted in every section, and EGFR immunostaining was graded as 1+ = 0–25%, 2+ = 25–75%, and 3+ = >75% of cells with positive staining.

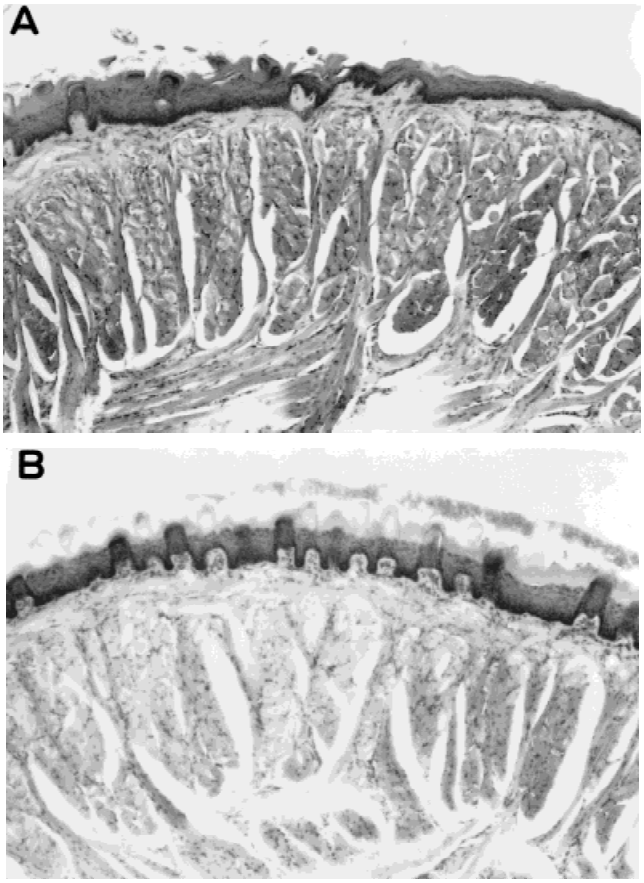

Fig. 1. Microscopic appearance of left tongue margins in group A **(A)** and group B **(B),** showing moderate epithelial dysplasia.

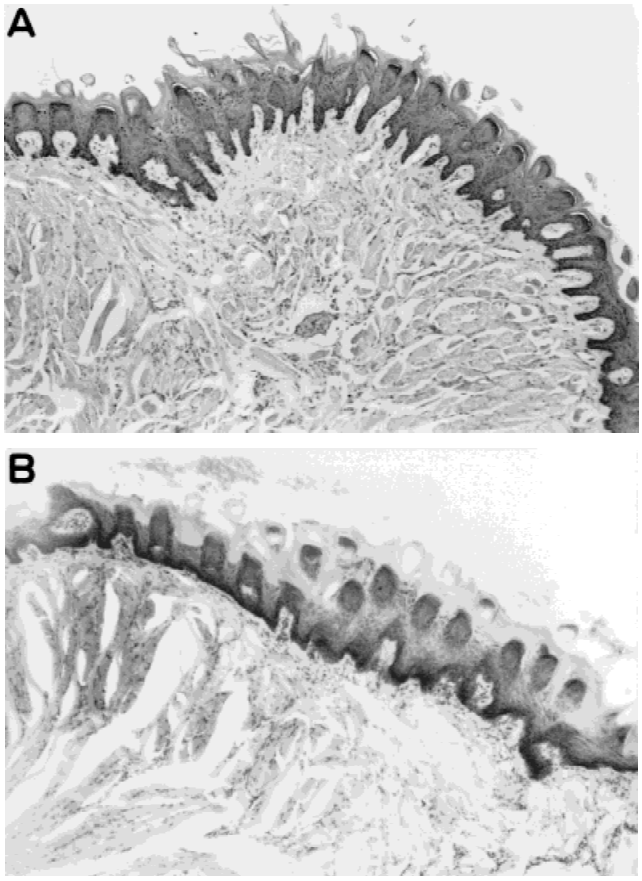

Fig. 2. Microscopic appearance of right tongue margins in group A **(A)** and group B **(B),** showing severe epithelial dysplasia.

RESULTS

Macroscopic and Microscopic Appearance

All mouse tongues in each group showed white patches with red areas. No inflammation or necrosis was observed.

In group C, the epithelia of mouse tongues showed mild to moderate dysplasia, and the appearances of both margins were similar. In group A and group B, the epithelia of the left margins showed mild to moderate dysplasia, similar to group C (Fig. 1). However, severe epithelial dysplasia was observed in every right tongue margin of group A and group B (Fig. 2). There was no obvious difference between these two groups. Malignant lesions were not detected in any group.

Immunohistochemistry of PCNA, EGFR, and p53

PCNA-positive cells were observed in all sections (Fig. 3). Positive nuclei were distributed in the basal and parabasal cells of the epithelium. PCNA LIs are given in Table 1. There was a significant difference in PCNA LIs between the left and right margins in group A ($P = 0.0006$) and group B ($P = 0.0273$). However, there was no significant difference in PCNA LIs between group A and group B with regard to the left margins ($P = 0.725$) or right margins ($P = 0.383$).

EGFR immunostaining at the 1+ ~ 2+ level was observed throughout both the parabasal and basal epithelial layers in group C, and the left margins of group A and group B. However, 3+ EGFR immunostaining was identified in all the right margins of group A and group B (Fig. 4); positive cells were observed throughout the entire lesion of dysplastic epithelium, and no obvious difference was found between group A and group B. Immunostaining was predominantly localized to the cell membrane, although some cytoplasmic staining was also seen.

Only one positive p53 immunostain was detected in the left tongue margin of group A (Fig. 5). The other sections showed negative p53 staining.

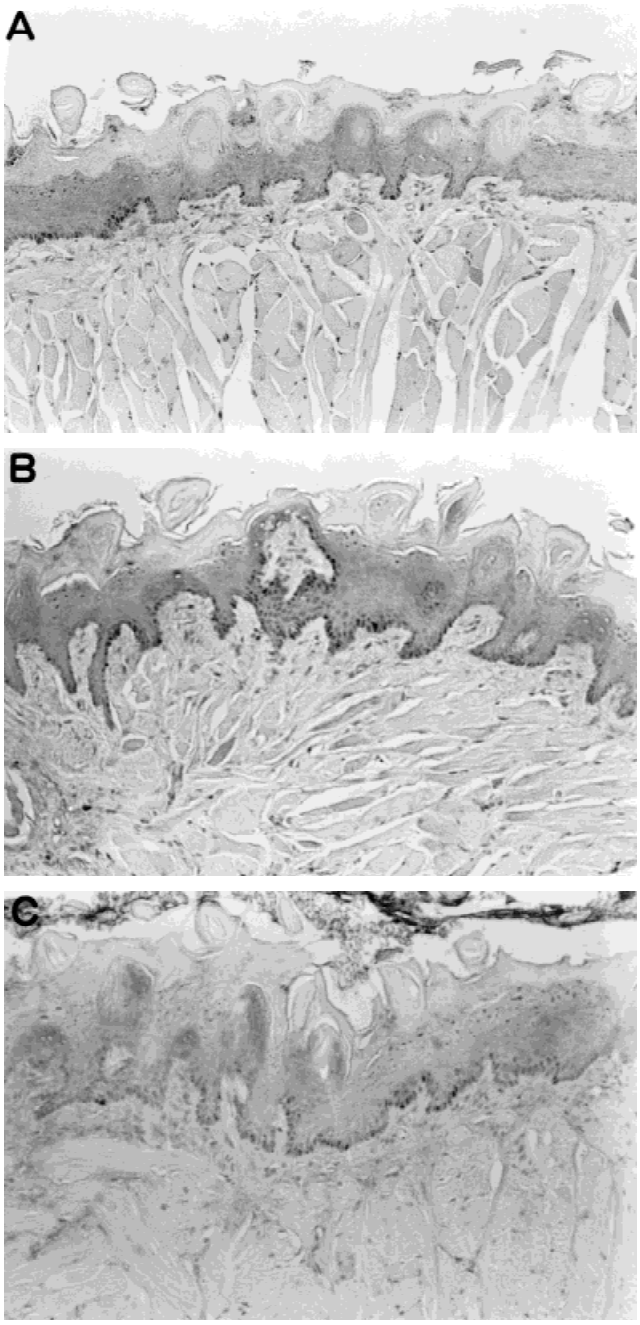

Fig. 3. PCNA immunohistochemical staining in the left tongue margins of group A **(A)** and right tongue margins of group A **(B)** and group B **(C)**. The positive nuclei were distributed in the basal and parabasal cells of the epithelium.

TABLE 1. Proliferating Cell Nuclear Antigen Labeling Indices

	PCNA LIs (mean ± SD)		
	Group A	Group B	Group C
Right margins	41.53 ± 8.23	43.39 ± 9.43	33.76 ± 5.98
Left margins	33.08 ± 6.38	35.06 ± 5.15	32.84 ± 6.32

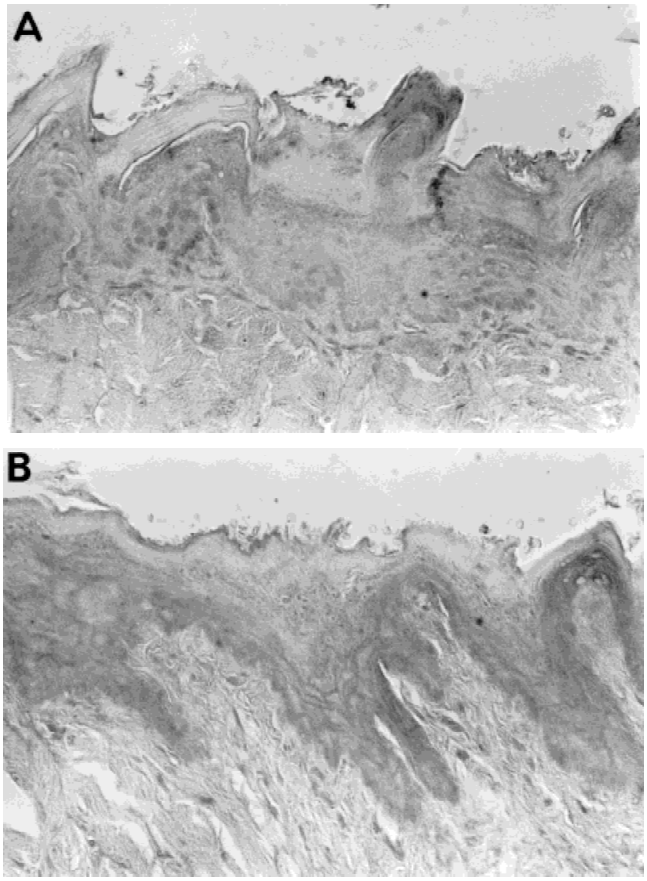

Fig. 4. The expression of EGFR in the left tongue margins of group B **(A)** and right tongue margins of group A **(B).** Positive immunostaining was predominantly localized to the cell membrane.

DISCUSSION

Laser and scalpel incision have thus far been considered to be the most effective therapies for oral precancerous lesions. However, cancer promotion caused by laser and scalpel treatment has been reported in previous studies [8–12]. The hamster buccal pouch of a precancerous model induced with 9,10-dimethyl-1,2-benzanthracene (DMBA) was used in previous studies for detecting the promotion of laser and scalpel incision [8,10]. However, the cheek pouch is not subjected to the same environmental influences generally found in the oral cavity [23], and some studies have demonstrated that the leukoplakia of the tongue showed the highest incidence of malignant transformation [1,4,6].

A precancerous model of mouse tongue induced by oral administration of water-soluble carcinogen 4NQO in drinking water was employed in

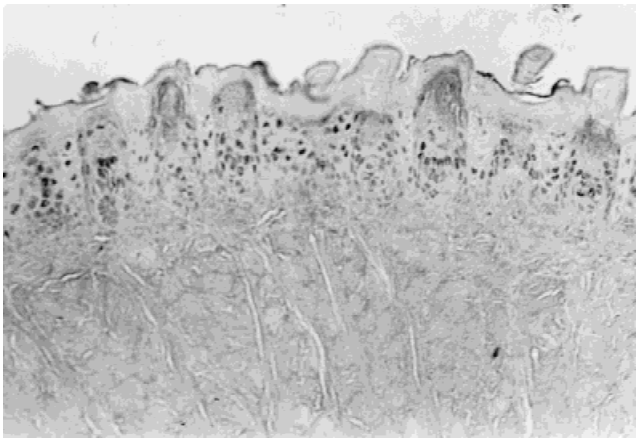

Fig. 5. Only one case of positive p53 immunostaining was detected in the left tongue margin of group A.

the present study. The morphologic appearance of tongue lesions developed by this method showed similarity to that of human lesions [23–25].

In the present study, no cancerous lesion was observed in any mouse, but dysplasia involving the right tongue margins brought about more serious findings than those of the left tongue margins in both group A and group B. No significant histologic difference was found between group A and group B. This result suggests that CO_2 laser and scalpel incision have similar promotional effects on malignant transformation.

PCNA is associated with DNA synthesis and cell proliferation, and it has been demonstrated to be a useful biomarker for better understanding of multistep carcinogenesis in cancers of the head and neck [14,15,26]. In the present study, the PCNA LIs of the right tongue margins were significantly higher than those of the left tongue margins in both group A and group B, and there was no significant difference that CO_2 laser and scalpel incision have similar effects on promoting cell proliferation in premalignant lesions.

Growth factors have been shown to play an important role in different phases of wound healing [27]. After laser or scalpel surgery, growth factors are released and stimulate epithelial cells to migrate and proliferate [13,27]. However, these growth factors also act as cancer promoters in initiated fields [16–19], as do most mitogenic agents. EGF is a representative growth factor which elicits a wide range of rapid and delayed responses through binding to high-affinity EGFR [16]. Increased EGFR immunostaining intensity was noted with higher degrees of dsyplasia, and indicates association with malignant transformation [18,19]. EGFR expression has therefore been suggested as a possible marker of malignant transformation. A previous study reported that epithelia showing two-thirds or greater thickness of positive EGFR had a high risk of maligant progression [18]. The present study showed that the EGFR staining intensity of the right tongue margins was higher than that of left tongue margins in both group A and group B, and that all the epithelia of the right tongue margins in both groups showed greater than two-thirds EGFR. No evident difference of EGFR was found between group A and group B. This result may indicate that both CO_2 laser and scalpel incision have similar potential concerning the release of growth factors, and that larger amounts of growth factors may promote premalignant lesions to malignant transformation.

The p53 gene, which encodes 53 KD nuclear phosphoprotein, is a tumor-suppressor gene located on chromosome 17p13, and is a cell regulator [20]. It has been suggested that p53 alteration may occur in the very early phases of carcinogenesis [19–21]. Premalignant lesions of the human head and neck frequently overexpress p53 [20]. In contrast, it is reported that only 3% of mouse premalignant lesions proved to be p53-positive [28]. Some studies have determined that the normal epithelia adjacent to malignant or premalignant lesions showed overexpression of PCNA, EGFR, and p53, and had high risk of tumor development [22,29–32]. Based on these reports, although the alteration of p53 expression was not detected in our study, severe epithelial dysplasia and significant increase of PCNA and EGFR expressed after CO_2 laser and scalpel incision are suggestive of a high risk of malignant transformation.

We conclude that CO_2 laser and scalpel incision appear to have similar promotional effects on premalignant lesions.

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